Biological relevance of Cytomegalovirus genetic variability in congenitally and postnatally infected children. [*Dell'Oste V, *Landolfo S, co-corresponding authors]

Biological relevance of Cytomegalovirus genetic variability in congenitally and postnatally infected children

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ABSTRACT

Background. Human cytomegalovirus (HCMV) is the leading cause of congenital infections resulting in severe morbidity and mortality among infected children. Although the virus is highly polymorphic, particularly in genes contributing to immune evasion, the mechanisms underlying its genetic variability and pathogenicity are only partially understood.

Objectives. We aimed to characterize different HCMV clinical strains isolated from 21 congenitally- or postnatally-infected children for *in vitro* growth properties and genetic polymorphisms.

Study design. The growth of various HCMV isolates was analyzed in different cell culture models. Genetic polymorphism was assessed by genetic and phylogenetic analysis of viral genes involved in virulence (UL144, US28, and UL18), latency (UL133-138), or drug resistance (UL54 and UL97).

Results. Here, we report a high degree of genetic and phenotypic diversity in distinct HCMV clinical isolates, as shown by their *in vitro* growth properties. In particular, HCMV isolates displayed the highest degree of genetic variability in the UL144 gene, where we were able to define four distinct genotypes within the cohort based on UL144 heterogeneity. Lastly, among all isolates we were able to identify 36 mutations in UL54 and 2 in UL97.

Conclusions. Our findings indicate that surprisingly high levels of genetic HCMV variability correlate with a high degree of phenotypic polymorphism, which in turn might differentially influence the growth, fitness, and drug susceptibility of HCMV.

Keywords: human cytomegalovirus (HCMV), congenital infection, clinical isolates, genetic variability, viral phenotypes.

HIGHLIGHTS

- HCMV clinical isolates possess different *in vitro* replication properties
- They all share the same cell tropism, albeit showing different morphological patterns
- For the most variable gene, UL144, four distinct genotypes were defined
- In each HCMV isolate, we identified 36 mutations in UL54 and only 2 in UL97

1. Background

Human cytomegalovirus (HCMV), a double stranded DNA herpesvirus, is the most frequent cause of congenital malformations worldwide, resulting in neurodevelopmental delay, foetal or neonatal death, and most frequently sensorineural hearing loss [1–3]. It is characterized by a large genome encoding a wide range of gene products, endowed of effective immunomodulatory activity [4–6]. For instance, different viral genes encoding tumor necrosis factor-alpha (TNF-α) receptor (UL144), α-chemokines (UL146-147), β-chemokine receptor (US28) are potential virulence factors associated with severe congenital HCMV infection [7,8].

Mounting evidence suggests that HCMV can be highly polymorphic, among and within hosts [9–13], with a high level of intrahost variability comparable to that of RNA viruses. It has been demonstrated that new mutations occur every time that the virus infects a new host, thereby giving rise to a unique viral strain for each infected individual. HCMV infection triggers indeed a selection event where a new genotype becomes dominant due to the selective pressure of the immune response [10]. Another possible explanation of this gap comes from the observation that both viral and host factors can contribute to the onset of HCMV genome mutations, thus fostering virus genetic drift during infection [14,15].

HCMV genetic variability, an emerging issue in drug resistance, represents another major obstacle on the way to predicting clinical outcomes of HCMV congenital infections. Currently, the only antiviral therapy available relies on nucleoside analogs, such as ganciclovir (GCV) and valganciclovir (VAL-GCV) [3,16]. In this regard, evidence from adult transplanted patients has shown that DNA polymerase (UL54) and viral phosphotransferase (UL97), two highly polymorphic HCMV genes, seem to play a role in drug resistance against GCV [17]. However, further research is clearly needed to fill the lack of information on congenitally HCMV infected children.

2. Objectives

Against this background, the aim of our study was to characterize the *in vitro* phenotype and the degree of genetic polymorphism of HCMV virions freshly isolated from congenitally or postnatally infected children, focusing on genes encoding potential virulence factors, such as UL144, US28, UL18, or contributing to viral latency, such as UL133-138. In addition to the aforementioned immunomodulatory genes, we also analyzed UL97 and UL54 to assess the emergence of drug resistant strains within the enrolled group of patients. Finally, we investigated a potential association between genotype and viral fitness.

3. Study design

3.1. Patients and samples

Twenty-one children diagnosed with congenital or postnatal HCMV infection were recruited at the Neonatal Unit of the University of Turin from 2015 to 2017. Infection diagnosis was based on RT-PCR HCMV DNA detection in patients' urine and blood samples. Urine samples were collected during the admission medical examination. The Neonatal Unit created a detailed database on clinical and pathological characteristics of recruited patients (indicated as P), summarized in Table 1. All patients were evaluated for neurobehavioral development, growth parameters, cerebral ultrasound, sight and hearing, antiviral and supportive therapy [18]. In addition, they were subjected to a follow up of one year of clinical and neurobehavioral tests for asymptomatic patients, and two years for symptomatic patients along with 6 years of audiology tests.

3.2. Cells and viruses

Primary human foreskin fibroblasts (HFF, American Type Culture Collection, ATCC SCRC-1041™), human retinal pigment epithelial cells (ARPE-19, ATCC CRL-2302™), and

human umbilical vein endothelial cells (HUVEC) were cultured as previously described [19]. For HFF infection with HCMV clinical isolates, urine samples were primarily inoculated in HFF in order to boost the infected cell population. The isolates were then propagated until approximately 60% of cells demonstrated a cytopathic effect. All isolates were used before passage 3 in order to avoid cell culture adaptation [20,21].

3.3. Viral replication analysis

The replication of cell-associated isolates was quantified by focus expansion assay (FEA), as previously described [22]. Plaque area was calculated using ImageJ software. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows.

3.4. Immunofluorescence microscopy

Indirect immunofluorescence analysis was performed at 72h pi as previously described [23]. The following primary antibodies were used: rabbit polyclonal anti-human von Willebrand factor (vWF) (Sigma-Aldrich), anti-IEA (immediate early antigen; produced in Santo Landolfo's laboratory, University of Turin [24]), mouse monoclonal anti-IEA, UL44 (Virusys Corporation), and pan cytokeratin (Sigma-Aldrich). Signals were detected using goat anti-rabbit or goat anti-mouse conjugated secondary antibodies (Life Technologies). Images were taken with a 40× objective by fluorescence microscope Olympus IX-70, equipped with cellSens Standard - Microscopy Imaging Software (Olympus), and ImageJ software was used for image processing.

3.5. DNA sequencing

Genomic DNA was extracted from infected cells by heating the cell with lysis buffer (1.25 M NaCl, 62 mM Tris-Cl pH 8.0, 9 mM EDTA pH 8.0, 0.5% SDS) for 15 min, followed by ammonium acetate/chloroform treatment, and the target genes were amplified using Q5

High-Fidelity DNA Polymerase (New England BioLabs). Primers designed on human herpesvirus 5 strain Merlin sequence (NC_006273.2) and PCR conditions are listed in Table 2. The amplified products were purified and used for Sanger sequencing (Eurofins Genomics).

3.6. Phylogenetic analysis

Nucleotide sequences were multiple-aligned to match homologue regions along Merlin reference genome (NC_006273) or along the most similar reference genomes. The alignment was performed using Clustal W, included into Geneious software 9.1, and each gene evolutionary model was selected using jModelTest 2.1.7 [25]. Gene sequences were concatenated, and the phylogenetic tree was reconstructed using a Bayesian approach (MrBayes 3.2.5) [26]. The tree was visualized with FigTree 1.4.2 software (Tree Figure Drawing Tool Version 1.4.2 2006-2014, University of Edinburgh). Robustness of the internal nodes was reported as a posterior probability calculated on the consensus of all the equally probable topologies obtained by the heuristic search. Recombination events were evaluated by using DualBrother plugin in Geneious software [27], SpliTree [28] and SimPlot [29], considering both single genes and concatenated alignments. Association between concatenated tree topology and clinical parameters was investigated using BaTS algorithms evaluating Association Index (AI), Parsimony score (PS) and monophyletic clade (MC) size statistics.

3.7 Identification of mutations associated with antiviral drug susceptibility in HCMV clinical strains

To ascertain whether the *in vitro* growth variability of HCMV clinical isolates correlated with different degrees of drug susceptibility, we used the web-based search tool mutation resistance analyzer (MRA) is a platform linking identified HCMV drug resistance mutations to specific phenotypes (http://www.informatik.uni-ulm.de/ni/mitarbeiter/HKestler/hcmv) [30]. Detected mutations are then run through a

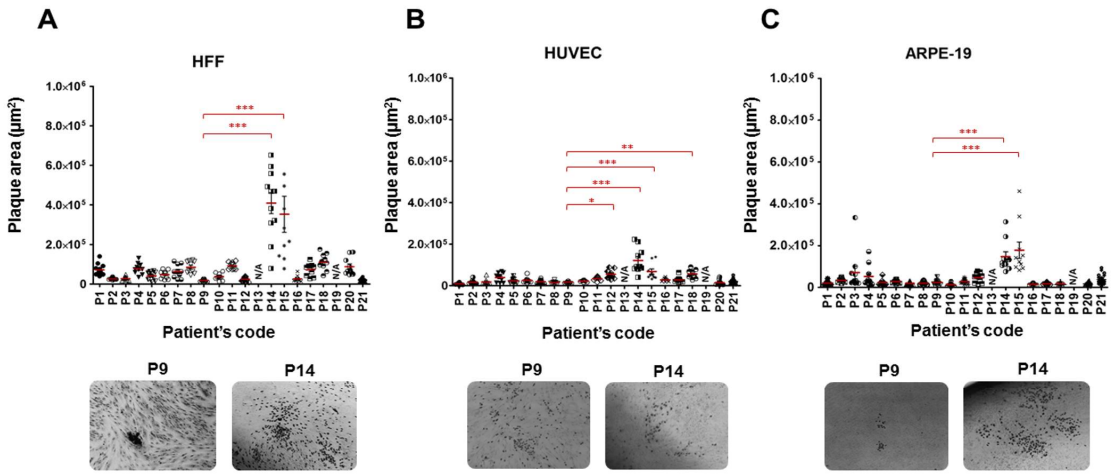
151 regularly updated database containing previously published UL97 and UL54 mutations and the
152 corresponding *in vitro* drug susceptibility phenotypes.

153 **4. Results**

154 *4.1. Phenotypic characterization of HCMV clinical strains*

155 First, we carried out phenotypic characterization of HCMV isolates from all patients
156 (P), with the exception of P13 and P19, in HFF. Interestingly, we observed a remarkable
157 variation of fibroblast growth properties among the various isolates, with a high value range of
158 infected foci per well (Table 3).

159 Since cell-free virus transmission is typified by a comet tail phenotype, while cell-
160 associated transmission is characterized by plaques with well-defined edges [22], we sought to
161 determine the transmission pattern of different HCMV clinical isolates by defining plaque
162 morphology. Furthermore, to quantify HCMV replication, we calculated the relative plaque
163 area in HCMV infected HFF. We found that among all isolates, P14 and P15 were those
164 displaying the most aggressive/fast-replicative behavior (Fig. 1A upper panel). These results
165 were also supported by virus plaque morphology analysis showing that P14 displayed larger
166 and comet shaped plaques compared to P9 (Fig. 1A, lower panel). We observed the same
167 infection pattern in HUVEC and ARPE-19 (Fig. 1B and C). Accordingly, in these two cell
168 lines, plaque morphology and area analysis revealed a great heterogeneity (Figure 1B, C) even
169 though the extent of viral growth in HFF did not exactly mirror that seen in HUVEC and ARPE-
170 19. Indeed, in HUVEC P12 and P18 showed a statistically significant larger plaque area
171 compared to that of P9, albeit to a lower extent with respect to P14 and P15, indicating that
172 HCMV replication not only depends on its genetic background, but also on cell environment.



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174 **Fig. 1. Replication properties of HCMV clinical isolates.** Viral replication was analyzed by focus
175 expansion assay (FEA). Serial dilutions of HFF (A), HUVEC (B), and ARPE-19 (C) infected by clinical
176 isolates were cocultured with an excess of uninfected HFF for 5 days. Monolayers were then fixed, and
177 infected cells were traced by antibodies against HCMV immediate early antigen (IEA), followed by
178 immunoperoxidase assay. Infectious foci were defined as clusters of at least three infected cells, starting
179 from single late-stage infected cells. *Upper panel.* Plaque areas were calculated using ImageJ software.
180 The red bars represent mean values. Differences were considered statistically significant for *, P< 0.05;
181 **, P< 0.01; ***, P< 0.001 (one-way ANOVA followed by Bonferroni's post-tests, GraphPad Prism
182 version 5.00 for Windows, for comparison of all isolates *versus* P9). *Lower panel.* Representative
183 infectious foci of clinical isolates 9 (P9) and 14 (P14) are shown.

184 *4.2. Definition of the endothelio- and epithelio-tropic phenotype of different HCMV isolates*

185 Next, we assessed HCMV isolates for viral growth. The FEA in HUVEC and ARPE-
186 19 revealed that all isolates retained their endothelial and epithelial tropism (Fig. 1 B, C). To
187 rule out the possibility that inoculated infected HFF could overgrow to form infected foci
188 within HUVEC and ARPE-19, we performed an immunofluorescence double staining for von

Willebrand factor (vWF) and pan cytokeratin (Fig. 2 A and B). Based on HCMV aggressiveness, epithelial cell infection resulted in two distinct morphological phenotypes (Fig. 2). Whereas fast-replicative isolates, such as P14, formed enlarged flower-shaped syncytial foci, slow-replicative isolates, such as P9, were only visible as single mononucleated infected cells (Fig. 2A). These dual phenotypes were not observed in HUVEC, suggesting a distinct replication pattern among different cell lines and viral isolates (Fig. 2B).

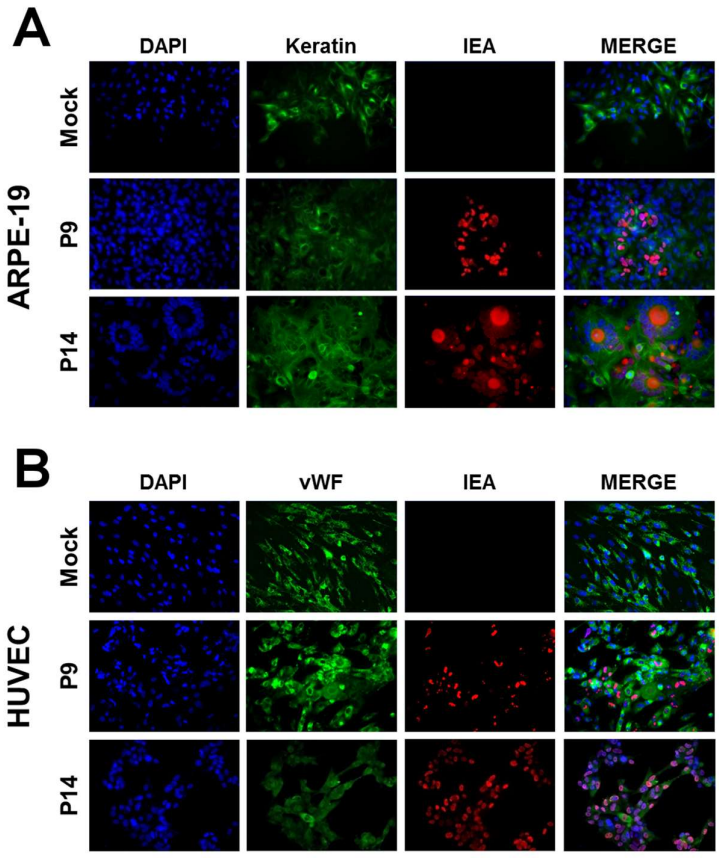


Fig. 2. Definition of the endothelio- and epithelio-tropic phenotype of different HCMV clinical isolates. ARPE-19 (A) and HUVEC (B) were cocultured with an excess of HFF infected with representative HCMV clinical isolates (P9 and P14) or mock infected. Cells were fixed 72 h later for immunofluorescence analysis to detect HCMV immediate early antigen IEA (red) and the inherent cell markers: endothelial vWF (green) or epithelial keratin (green). Cell nuclei were counterstained with

4',6-diamidino-2-phenylindole (DAPI) (blue). Images were taken with a 40× objective by fluorescence microscopy. The most representative infectious foci are shown.

4.3. Genetic characterization of HCMV clinical strains

To determine whether phenotypic changes were accompanied by alterations at the DNA level, we performed comparative analysis of a set of genes encoding potential virulence factors (i.e. UL144, US28, UL18), or contributing to viral latency (i.e. UL133-138), or associated with drug resistance (i.e. UL54, UL97) (Table 2). For each region/gene, the sequences were aligned and, based on the best GTR+Γ evolutionary models, the Bayesian trees were drawn (Fig. S1-S6). Given that HCMV often shows recombination events [31], the alignment was used to create a split network (Fig. 3A), and a set of reference sequences was included. Statistically significant evidence of recombination was identified along the concatenated alignment (Φ $p < 0.05$) (Fig. 3A). Moreover, the SimPlot showed a great heterogeneity (Fig. 3B), reaching the highest variability within the UL144 gene (Fig. 3C), found exclusively in clinical HCMV strains.

Remarkably, UL144 amino acid sequence alignment from all HCMV isolates defined four prevalent subgenotypes, namely A, B, C, and A/B (Fig. 4B). The majority of sequences matched with genotype B, whereas those from the isolates P1, P12, and P20, closely related to the Merlin reference strain, were classified under genotype A. Furthermore, sequences derived from the P5, P18 and P21 isolates matched with genotype C, whereas only the P3-derived amino acid sequence was listed under genotype A/B. Interestingly, we noticed that both the P14 and P15 fast-replicative strains belonged to genotype B, indicating that the viral genetic background can indeed determine viral fitness.

Noteworthy, considering both nucleotide (Fig. 3C) and amino acid sequences (Fig. 4A), the most important finding deriving from the SimPlot is that the 5' region is the key to

discriminate the four genotypes. However, despite having a different genotype, we observed that almost all cysteines were conserved along the alignment, suggesting that distinct HCMV isolates may share a similar viral protein folding.

Finally, no statistically significant associations were found between the concatenated tree topology and any clinical parameter reported in Table 1, considering both global (AI and PS) and local (MCs) association parameters.

GenBank accession numbers of all sequences are reported in Table S1.

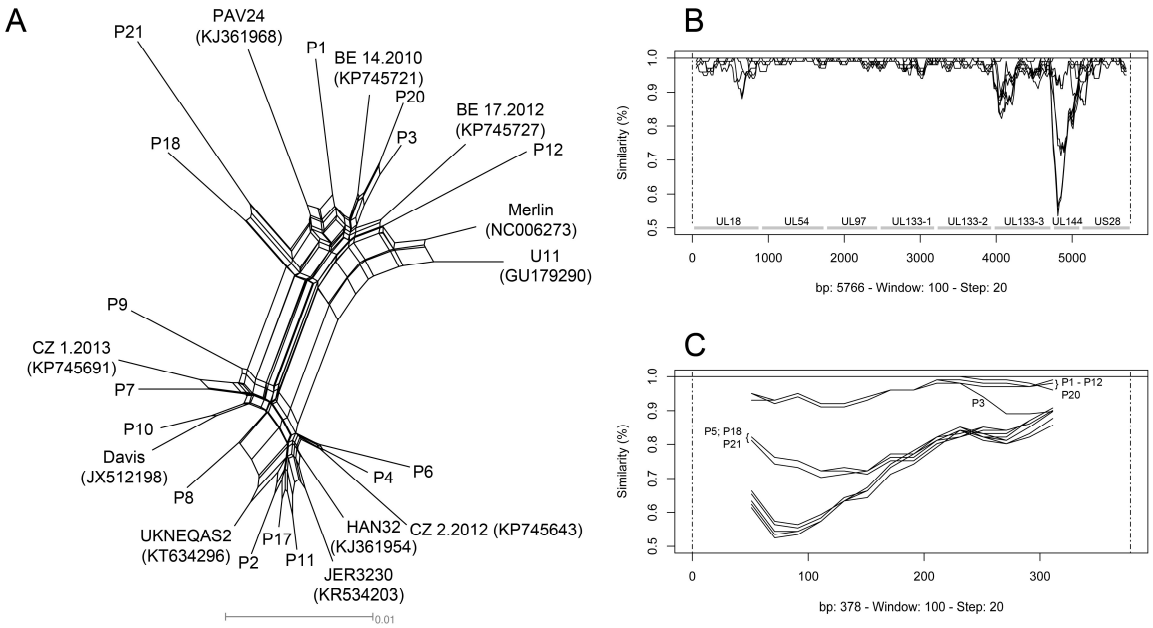
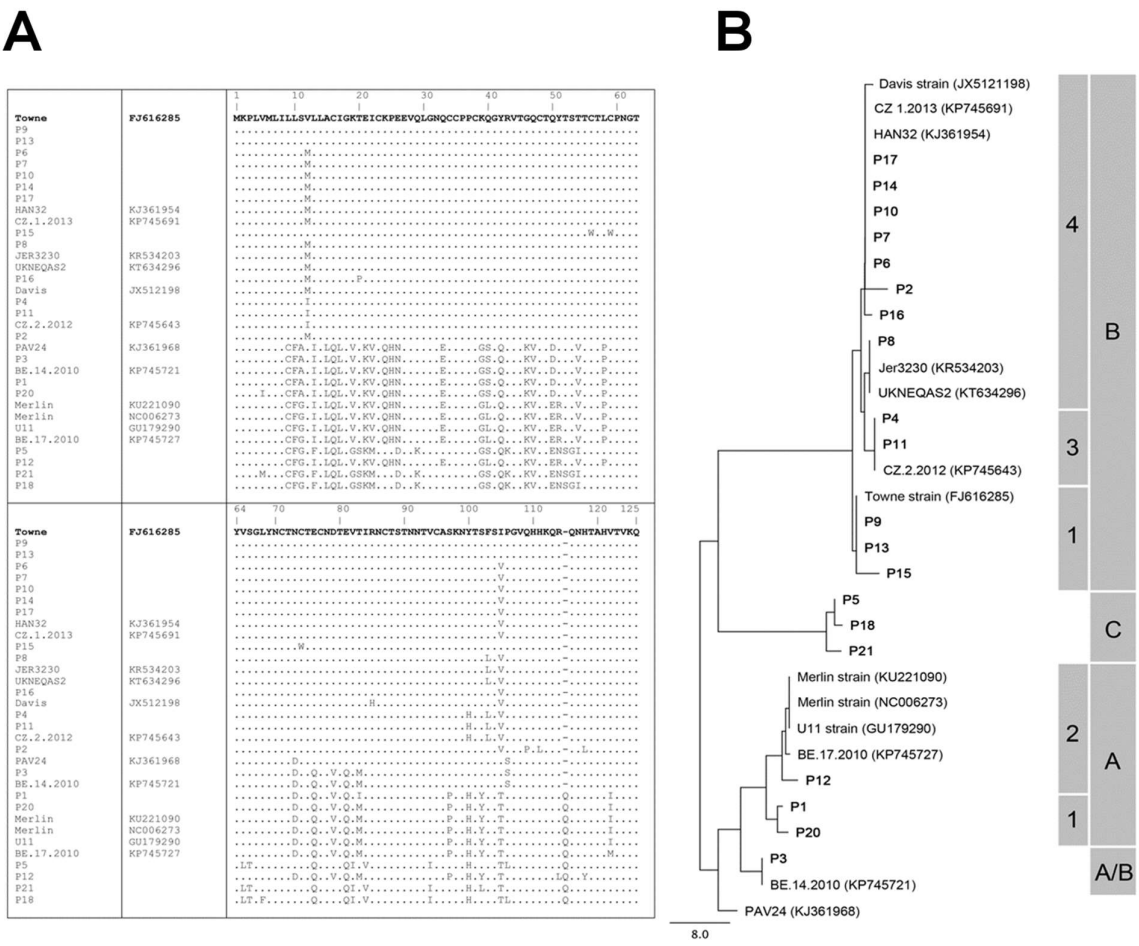


Fig. 3. Genetic analyses on HCMV clinical isolates. (A) Split network; (B) SimPlot of concatenated alignment; (C) SimPlot of UL144 alignment; mean nucleotide diversity among samples = 86.59% (range 73.0% - 100.0%). For both the SimPlots, Merlin strain was used as reference sequence.



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237 **Fig. 4. UL144 gene analysis.** Amino acid alignment (A) and neighbor joining tree (B), based on amino
238 acidic sequence alignment, are reported. Dots indicate identical residue. Genotypes are indicated for
239 each tree clade.

240 *4.4. Antiviral drug susceptibility of HCMV clinical strains*

241 Complete sequences of UL54 and UL97 from HCMV isolates were uploaded in MRA
242 and compared to the wild-type sequence of the drug-sensitive HCMV strain TB40-BAC4
243 [32,33]. MRA identified 36 mutations in the UL54 gene associated with genetic polymorphism
244 previously published [34–39], whereas only two mutations were detected in UL97, probably
245 due to a major gene stability (Figure 5A and B) [35,40–43].

Interestingly, drug resistance-associated mutations varied among patients, especially in UL54 and to a lesser extent in UL97 (Figure 5A and B).

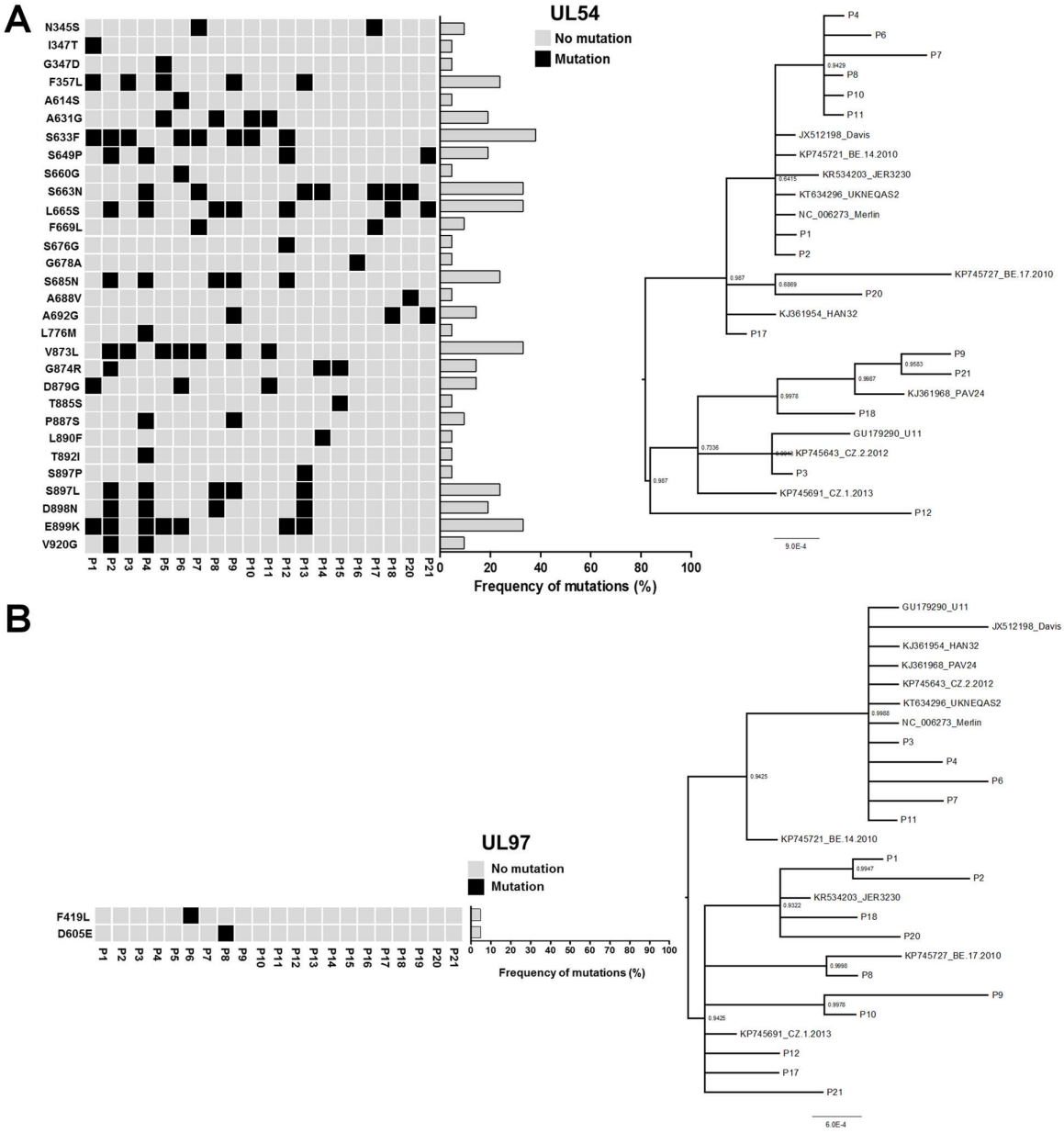

Fig. 5. Antiviral susceptibility testing. The heterogeneity of UL54 (A) and UL97 (B) genes is reported. *Left panel.* The heat maps show the presence and the positions of amino acidic changes, and the histograms show the frequency of each modification. *Right panel.* The Bayesian tree describes the genetic relationship among samples based on nucleotide alignment.

5. Discussion

The clinical and biological relevance of HCMV genetic variability in congenitally and postnatally infected children has been the focus of intense research over the past few years. In this study, we sought to determine whether and to what extent the differences in gene composition affected viral fitness. For this purpose, we enrolled 21 pediatric patients with confirmed congenital or postnatal HCMV infection. We evaluated the degree of genetic polymorphism of HCMV clinical strains by genetic and phylogenetic analyses, primarily focusing on viral genes involved in virulence, latency, and drug resistance. In parallel, we ran an extensive *in vitro* analysis of all clinical isolates to characterize viral growth properties and viral tropism in fibroblasts, endothelial and epithelial cells. Our results suggest that HCMV clinical isolates possess phenotypic differences as judged by both viral dissemination rate and replication properties, which define the extent of strain aggressiveness. Particularly, the two strains P14 and P15 were the most aggressive and fast-replicative ones because they could give rise to infectious foci characterized by comet-shaped plaques, typically observed in laboratory strains [22]. Altogether, these results support the hypothesis that HCMV heterogeneity may have an impact on viral fitness, influencing both viral dissemination rate and replication properties.

The reliability of such results could be inferred from the different cell lines employed for virus propagation and their low passage number ($\leq$ 3) to ensure that no cell-culture adaptation had occurred [20,21]. All clinical isolates in the recruited group of patients were able to infect both epithelial and endothelial cells displaying no difference in their cell tropism, while they displayed a unique morphological pattern in cells infected with fast-replicative isolates. Interestingly, the enlarged flower-shaped syncytial foci typical of epithelial, but not endothelial, cells obtained with the most aggressive strains were similar to those observed by Tandon and coworkers in HFF infected with UL96-deleted Towne bacterial artificial

chromosome (ΔUL96BAC) [44], which could be partly ascribed to different maturation patterns between isolates.

The analysis of specific HCMV genome regions suggests that genetic variability among HCMV isolates may impact viral fitness. Indeed, here we report enhanced sequence diversity, identified thanks to the 5' region of UL144 alignment used as a discriminatory criterion. Interestingly, the high degree of nucleotide heterogeneity mirrors in the amino acidic sequence, indicating a considerable difference among UL144 genotypes. This is interesting, as UL144 is a potent NF-κB activator [45] that plays a role in virus-mediated immune evasion [46,47]. This high heterogeneity of UL144 strongly influenced the split tree configuration based on the concatenated alignment, highlighting the role of this gene in the description of genetic relationships between CMV isolates. The same sample clustering has been demonstrated for both UL54 and UL97 genes, known to be involved in antiviral drug resistance. In both cases, the similarity among samples was high even though a number of previously reported mutations were identified in UL54 and to a lower extent in UL97. However, all treated patients so far responded to valganciclovir therapy. Nonetheless, we are not able to fully exclude the possibility that the reported mutations might have an impact on the antiviral therapy over a prolonged time.

It is highly likely that multiple strains of CMV are present in each patient of our group of patients, similar to cases being previously extensively reported in the literature [12]. However, we used an approach based on the Sanger sequencing of PCR products obtained from cell culture. The sensitivity of this method is strongly dependent on the relative frequency of viral variants. In particular, low-abundance viral populations are likely to be missed and the overall viral diversity to be underestimated. Thus, although sequence electropherograms did not show clear evidence of multiple signals (i.e., double peaks and/or high background signal), we can not exclude the presence of mixed infections. Further investigations can be carried out

303 to evaluate the role of mixed infections in pediatric infected patients, including PCR product
304 cloning strategies or next generation sequencing approaches.

305 The limits of our study include the small number of the recruited patients, the short
306 collection period (two years) in the limited geographic area. Besides, not all the analysis were
307 available for all the samples, f.i. propagation and isolation of the virus failed for P13
308 (simultaneous presence of pathogenic bacteria and yeast in the patient's urine, since patient 13
309 was presented with severe sepsis at admission) and P19 (low viral load in urine sample).

310 In conclusion, our study may suggest that genotypic variability is associated with *in*
311 *vitro* phenotypic diversity in HCMV clinical strains isolated from a group of congenitally and
312 postnatally infected patients. In addition, our results indicate that genetic polymorphisms
313 across the UL54 genome might play a role in multidrug resistance HCMV infection, pointing
314 to UL54 as a potential therapeutic target to consider when treating congenital HCMV disease.
315 This is, to our knowledge, the first detailed analysis that tries to associate in the same cohort of
316 patients' genetic polymorphism and viral fitness. Although the results achieved so far do not
317 allow any definitive conclusion, it emerges that a strong genetic HCMV variability is reflected
318 in a remarkable phenotypic polymorphism that could affect virus growth properties and *in vivo*
319 fitness.

320

321 **Competing interest**

322 None declared.

323 **Authors' contribution**

Study design: SL, VDO, MDA; laboratory analyses: GG, VDO, MB; patients' management and clinical data collection: EB, AL, AC; statistical/phylogenetic analyses: LB and UA; manuscript writing: GG, VDO, SL.

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Ethical approval

This study was approved by the Research Ethics Committee of the University Hospital of Turin "A.O.U. Città della Salute e della Scienza di Torino – A.O. Ordine Mauriziano – A.S.L. TO1" (No 007816). Informed consent was obtained from parents of all study participants prior to the collection of demographic and clinical data, along with biological samples. The work was carried out in accordance with the Declaration of Helsinki.

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Tables

Table 1. Clinical-pathological characteristics of patients

<table>
<tr><th colspan="14">MOTHER</th><th colspan="4">FETUS</th></tr>
<tr><td colspan="2">Previous history of childbirths</td><td colspan="2">Infection discovery</td><td colspan="2">Type of infection</td><td colspan="2">Trimester of infection</td><td colspan="2">Fetal anomalies</td><td colspan="2">Viral load (amniotic fluid)</td></tr>
<tr><td rowspan="2">First born</td><td rowspan="2">71 %</td><td>0 = unsuspected during pregnancy</td><td>50%</td><td>1=first</td><td>47.6%</td><td>1st</td><td>14.3%</td><td rowspan="4">19%</td><td rowspan="2">Neg</td><td rowspan="2">9.5%</td></tr>
<tr><td>1= serology</td><td>50%</td><td>2=relapse</td><td>14.3%</td><td>2nd</td><td>33.3%</td></tr>
<tr><td rowspan="2">Not first born</td><td rowspan="2">29 %</td><td>2=ultrasound abnormalities (not IUGR)</td><td>0</td><td>3=acquired after birth</td><td>9.5%</td><td>3rd</td><td>19.1%</td><td>Pos</td><td>0%</td></tr>
<tr><td>3=IUGR</td><td>0</td><td>N/A[b]</td><td>28.6%</td><td>After birth</td><td>9.5%</td><td>N/A</td><td>90.5 %</td></tr>
<tr><td colspan="4">CHILDBIRTH</td><td colspan="10">NEWBORN</td></tr>
<tr><td colspan="2">Gestational age (mean week): 37.6</td><td colspan="2">Reanimation at birth: 14.3%</td><td colspan="2">Sex</td><td colspan="4">Birth weight (0=≥10°pc; 1=3-10 pc; 2=≤3°pc)</td><td colspan="4">Head circumference (0= ≥10°pc/ 1=<10°pc)</td></tr>
<tr><td colspan="4" rowspan="4">Children age at time of sampling (mean month): 2.5</td><td rowspan="4">F 47.6%</td><td rowspan="4">M 52.4%</td><td colspan="2">0</td><td colspan="2">61.9%</td><td rowspan="2">0</td><td rowspan="2">71.5%</td></tr>
<tr><td colspan="2">1</td><td colspan="2">9.5%</td></tr>
<tr><td colspan="2">2</td><td colspan="2">19%</td><td rowspan="2">1</td><td rowspan="2">19%</td></tr>
<tr><td colspan="2">N/A</td><td colspan="2">9.5%</td></tr>
<tr><td colspan="6">INSTRUMENTAL EXAMS ANOMALIES[c]</td><td colspan="8">LABORATORY ANALYSIS ANOMALIES[f]</td></tr>
<tr><td colspan="2">Hearing[c]</td><td colspan="2">Cerebral ultrasound[c]</td><td colspan="2">Cerebral MR[d] or CT[e,c]</td><td colspan="2">Platelets[c]</td><td colspan="2">Neutrophils[c]</td><td colspan="4">Hepatic functionality[c]</td></tr>
<tr><td>0</td><td>76.2%</td><td>0</td><td>52.3%</td><td>0</td><td>38%</td><td>0</td><td>80.9 %</td><td>0</td><td>76.2%</td><td colspan="2">0</td><td colspan="2">71.4%</td></tr>
<tr><td>1</td><td>14.3%</td><td>1</td><td>42.9%</td><td>1</td><td>28.6%</td><td>1</td><td>4.8%</td><td>1</td><td>9.5%</td><td colspan="2">1</td><td colspan="2">9.5%</td></tr>
<tr><td>N/A</td><td>9.5%</td><td>N/A</td><td>4.8%</td><td>N/A</td><td>33.4%</td><td>N/A</td><td>14.3 %</td><td>N/A</td><td>14.3%</td><td colspan="2">N/A</td><td colspan="2">19.1%</td></tr>
<tr><td colspan="6">VIRAL LOAD</td><td colspan="8">ANTIVIRAL THERAPY</td></tr>
<tr><td colspan="3">Viral load (urine)</td><td colspan="3">Viral load (blood)</td><td colspan="4">Valganciclovir (os) at time of sampling</td><td colspan="4">Valganciclovir (os) after sampling</td></tr>
<tr><td colspan="2">Neg</td><td>0%</td><td colspan="2">Neg</td><td>4.8%</td><td colspan="4" rowspan="4">4.8%</td><td colspan="4" rowspan="4">14.3%</td></tr>
<tr><td colspan="2">Pos</td><td>100%</td><td colspan="2">Pos</td><td>66.7%</td></tr>
<tr><td colspan="2">N/A</td><td>0%</td><td colspan="2">N/A</td><td>28.5%</td></tr>
<tr><td colspan="2">Mean (PFU/ml)</td><td>2364034.9</td><td colspan="2">Mean (PFU/ml)</td><td>43318.8</td></tr>
</table>

[a]IUGR: intrauterine growth restriction; [b]N/A: not available; [c]0: normal, 1: pathologic; [d]MR: magnetic resonance; [e]CT: computed tomography; [f]abnormal laboratory indicators: platelet count < 100,000/mm^3, neutrophils count < 1,000/mm^3, ALAT > 80 IU/l, conjugated bilirubin plasma level > 2 mg/dl and > 10% of total bilirubin, per os: oral administration.

Table 2. Primers and reaction conditions for amplifying full length HCMV ORFs.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing T (°C)	Amplicon size (bp)	Nucleotide position
UL144	TCGTATTACAAACC GCGGAGAGGAT	ACTCAGACACG GTTCCGTAA	62	736	182073-182808
UL18	CACACGGCTAAGA GGATACATC	GGTAAAGTAGT GCAGGAACGC	62	1146	23873-25017
US28	ACCGAGGGCAGAA CTGGTGC	TACGAAAAGAC CGAGGTAGCG	62	1145	225411-226525
UL133-138 A	AGAGTATGTCAGT CAAGGGC	GAGTAGATCGA GCAGAGAAT	52	1390	187371-188760
UL133-138 B	CGACACGGAGTTT GAGATTC	GCCCTTGACT GACATACTCT	58	1070	188741-189810
UL133-138 C	TCGGCAGCCGCTG TAGAGAT	GAATCTCAAA CTCCGTGTCG	62	990	189791-190780
UL54A	ATTCAGATCTCGTG CGTGTGCT	TGTGCCATGAT GATGGAAGG	58	1223	79737-80959
UL54B	TGGTGCGCGATCT GTTCAACAC	GCTTCCGAGAC CTCGCGATCCT	58	1399	78891-80289
UL97	GGACATGAGCGAC GAGAGCT	GTACGCGACAC GAGGACATC	58	774	142886-143659

521 **Table 3.** Growth properties of HCMV clinical isolates.

Patients' code	Mean No. of IEA positive foci/infected cell dilution		
	HFF	**HUVEC**	**ARPE-19**
P1	$8*10^2$	$5*10^3$	$4*10^2$
P2	$6*10^2$	$1*10^3$	$5*10^3$
P3	$2*10^2$	$1*10^3$	$7*10^3$
P4	$3*10^2$	$3*10^3$	$2*10^2$
P5	$5*10^2$	$2*10^3$	$3*10^2$
P6	$7*10^2$	$1*10^2$	$7*10^3$
P7	$2*10^2$	$1*10^2$	$3*10^2$
P8	$7*10^2$	$1*10^3$	$1*10^2$
P9	$2*10^2$	$1*10^5$	$8*10^4$
P10	$1*10^3$	$1*10^3$	$6*10^3$
P11	$4*10^3$	$3*10^4$	$3*10^2$
P12	$9*10^3$	$1.3*10^4$	$1*10^1$
P14	$3*10^1$	$1*10^2$	$1*10^2$
P15	$6*10^2$	$2*10^2$	$3*10^2$
P16	$5*10^2$	$1*10^1$	$1*10^2$
P17	$1.9*10^4$	$2.2*10^5$	$4.4*10^5$
P18	$4*10^4$	$2.7*10^5$	$7.9*10^5$
P20	$4*10^3$	$5*10^3$	$7*10^3$
P21	$9*10^2$	$5*10^2$	$1*10^2$

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